The American Journal of Pathology, Vol. 170, No. 2, February 2007

DOI: 10.2353/ajpath.2007.060339

Neurobiology

Gestational Vitamin B Deficiency Leads to Homocysteine-Associated Brain Apoptosis and Alters Neurobehavioral Development in Rats

Sébastien A. Blaise,* Emmanuelle Nédélec,* Henri Schroeder,† Jean-Marc Alberto,* Carine Bossenmeyer-Pourié,* Jean-Louis Guéant,* and Jean-Luc Daval*

From Unité 724, Institut National de la Santé et de la Recherché Médicale, Faculté de Médecine, Vandoeuvre-lès-Nancy; and Neurosciences Comportementales,† Institut National de la Recherché Agronomique, Faculté des Sciences, Vandoeuvre-lés-Nancy, France*

Hyperhomocysteinemia has been identified as a risk factor for neurological disorders. To study the influence of early deficiency in nutritional determinants of hyperhomocysteinemia on the developing rat brain, dams were fed a standard diet or a diet lacking methyl groups during gestation and lactation. Homocysteinemia progressively increased in the offspring of the deficient group and at 21 days reached 13.3 ± 3.7 μmol/L versus 6.8 ± 0.3 μmol/L in controls. Homocysteine accumulated in both neurons and astrocytes of selective brain structures including the hippocampus, the cerebellum, the striatum, and the neurogenic subventricular zone. Most homocysteine-positive cells expressed p53 and displayed fragmented DNA indicative of apoptosis. Righting reflex and negative geotaxis revealed a delay in the onset of integration capacities in the deficient group. Between 19 and 21 days, a poorer success score was recorded in deficient animals in a locomotor coordination test. A switch to normal food after weaning allowed restoration of normal homocysteinemia. Nevertheless, at 80 days of age, the exploratory behavior in the elevated-plus maze and the learning and memory behavior in the eight-arm maze revealed that early vitamin B deprivation is associated with persistent functional disabilities, possibly resulting from the ensuing neurotoxic effects of homocysteine. ***(Am J Pathol 2007, 170:667–679; DOI: 10.2353/ajpath.2007.060339)***

The sulfur-containing amino acid homocysteine (Hcy) is a metabolite of the essential amino acid methionine. It can either be remethylated to methionine by enzymes that require folate (vitamin B9) or cobalamin (vitamin B12) or be catabolized by cystathionine β-synthase (CBS) to generate cysteine.[1] Depending on genetic, dietary, and environmental factors, Hcy levels may vary considerably among individuals, and elevated plasma concentrations have been identified as a risk factor for a wide range of pathological situations, such as cardiovascular diseases, stroke, and neurodegenerative diseases.[2–5] Clinically, it is usually considered that in adults the normal range of Hcy concentration in plasma is 5 to 10 μmol/L, and plasma levels of 12 to 15 μmol/L Hcy have been associated with an elevated risk of cardiovascular and neurodegenerative diseases.[3,6] During pregnancy, severe complications have been associated with hyperhomocysteinemia, and the disturbance of maternal and fetal Hcy metabolism attributable to folate or vitamin B12 shortage has been shown to play a role in the etiology of recurrent early pregnancy loss, placental abruption, and preeclampsia, as well as intrauterine growth retardation and neural tube defects.[7–9] In addition, the prevalence of folate and vitamin B12 deficiency in the context of pregnancy is relatively high, especially in some countries from sub-Saharan Africa and North Europe,[10–12] and an elevated Hcy level in the mother is usually recorded during the last trimester of pregnancy.[13] Taken together, these observations encourage studies on long-term consequences of a dietary regimen that can influence homocysteinemia during the early period of life. Particularly little is known about the effects of increased homocysteinemia in fetuses and neonates on their subsequent brain development, especially at the functional level. Kruman and colleagues[14] have reported that exposure to low concentrations of Hcy can trigger apoptosis in cultured hippocampal neurons from the embryonic rat brain, suggesting that the amino acid may directly exert ad-

Supported by the Institut National de la Santé et de la Recherche Médicale (Inserm) and the 'Région Lorraine' (to S.A.B.).

Accepted for publication October 25, 2006.

Address reprint requests to Jean-Luc Daval, Ph.D., Inserm U.724, Faculté de Médecine, 9 Avenue de la Forêt de Haye, B.P. 184, 54500 Vandoeuvre-lès-Nancy, France. E-mail: jean-luc.daval@nancy.inserm.fr.

verse effects on neuronal cells. Likewise, cytotoxic effects of Hcy have been reported on rat astrocytes *in vitro*.[15] We have recently designed a new, clinically relevant model for studying the consequences of a vitamin B-deficient diet during gestation on Hcy metabolism in the newborn rat.[16] In the present study, we have used this model to investigate *in vivo* the long-term neurophysiological effects of an early deficiency in nutritional determinants of hyperhomocysteinemia in the developing rat.

Materials and Methods

Animal Treatments

Animal experiments were performed on Wistar rats (Charles River, l'Arbresle, France) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult female rats were maintained under standard laboratory conditions, on a 12-hour light/dark cycle, with food and water available *ad libitum*. One month before pregnancy, they were fed with either standard food ($n = 8$) (maintenance diet M20; Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) or with a diet lacking methyl donors, ie, vitamins B12, B2, folate, and choline ($n = 8$) (Special Diet Service, Saint-Gratien, France), according to Blaise and colleagues.[16] Choline was eliminated from the diet because the alternative pathway for the methylation of Hcy to form methionine is catalyzed by betaine-homocysteine methyltransferase (EC 2.1.1.5), which uses betaine, a metabolite of choline, as the methyl group donor. The assigned diet was constantly maintained until weaning of the offspring (ie, postnatal day 21); thereafter, all pups were fed with standard food until 80 days of age.

Sample Collection

In some experiments, blood samples were collected from the tail in living animals. Otherwise, rat pups were sacrificed by excess halothane at various developmental stages (2, 5, 21, or 80 days). Intracardiac blood samples were drawn for the measurement of plasma concentrations of vitamin B12, folate, and Hcy, and the whole brain, including cerebellum, was rapidly harvested. For immunohistochemical analyses, brains were immediately frozen in methylbutane previously chilled to −30°C and stored at −80°C. For metabolic studies, brain tissues were frozen in liquid nitrogen. They were then lysed at 4°C in 100 mmol/L potassium phosphate buffer (pH 7.3) containing protease inhibitors (protease inhibitor cocktail; Sigma Chemicals, St. Louis, MO), and proteins samples were quantified according to Bradford.[17]

Measurements of Vitamin B12, Folate, and Hcy Concentrations

Plasma and cerebral concentrations of vitamin B12 and folate were determined by radio-dilution isotope assay (simulTRAC-SNB; ICN Pharmaceuticals, Costa Mesa, CA).[13] Hcy concentrations were assessed by fluorescent polarization immunoassay (IMX system; Abbott, Fornebu, Norway).[12] In addition, vitamin B2 status has been assessed by measuring the erythrocyte glutathione reductase activation coefficient that corresponds to the ratio between enzyme activities determined with and without the addition of the co-factor FAD.[18]

Activities of CBS and Methionine Synthase (MS)

Each enzymatic activity was measured on tissue samples corresponding to 400 μg of total proteins. CBS activity was monitored by a method adapted from Taoka and colleagues,[19] and MS activity by a method adapted from Chen and colleagues,[20] as previously described by Blaise and colleagues.[16] In addition, in an attempt to evaluate the methylation status of brain cells, the concentrations of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) were monitored in brain tissue homogenates. For this purpose, the technique using solid-phase extraction and high-performance liquid chromatography was adapted from Delabar and colleagues.[21] Proteins were precipitated with 0.2 N $HClO_4$, before injection on the column (Lichrospher, 100 RP-C18, 5 μm, 250 × 4 mm ID). The mobile phase was applied at a flow rate of 0.75 ml/minute and consisted of 50 mmol/L sodium phosphate (pH 3.2), 10 mmol/L heptan sulfonate, and acetonitrile (10 to 20% from 0 to 20 minutes). Amounts of SAM and SAH were quantified by using an UV detector at 254 nm.

MS and CBS mRNA Assays

MS and CBS transcripts were analyzed as previously described by Blaise and colleagues.[16] Total RNAs were isolated from brain tissues using the RNeasy minikit from Qiagen (Courtaboeuf, France) and quantified by spectrophotometry. After control of RNA integrity, total RNA (2 μg) was reverse-transcribed using hexamer random primers and Omniscript (Qiagen) in a 20-μl-volume reaction of a buffered mixture containing 0.5 mmol/L dNTP. The cDNA products were diluted twofold, and 2 μl were used as a template for each amplification in polymerase chain reaction (PCR). Reactions were performed with *Taq*DNA polymerase (Gibco Invitrogen Corp., Cergy Pontoise, France) in 50 μl of a buffered solution containing 0.5 mmol/L dNTP, and 0.5 μmol/L of each primer. Separate PCR for MS, CBS, and L-27 (internal standard) amplification were undertaken with the following oligonucleotide primers: MS (NM 030864, 228-bp product), sense 5′-CAGATATAATTGGCTTGTCAGGACT-3′, anti-sense 5′-TTTCATCTAACAGCTGAGAACACAC-3′; CBS (NM 012522, 350-bp product), sense 5′-TTCCCCACATTACCACACAG-3′, anti-sense 5′-AGCACATCCACCTTCTCCAT-3′; and L-27 (NM 022514, 364-bp product), sense 5′-GCTGTCGAAATGGGCAAGTT-3′, anti-sense 5′CAAACTTGACCTTGGCCTCC3′. Conditions of PCR allowing to be in the exponential phase of PCR were for MS: number of cycles (n) = 31, annealing temperature (AT) = 61°C; for CBS, $n = 30$, AT = 56°C; for L-27, $n = 27$, AT =

62°C. Amplification products were analyzed by agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified by densitometry with ImageMaster 1D Primer software and ImageMaster apparatus (Amersham Pharmacia Biotech, Quebec, QC, Canada). Amplicons were selective in amplifying the desired target mRNA. Results were expressed relatively to the mRNA levels of the housekeeping gene *L27* for ribosomal protein.

Immunohistochemical Detection of Hcy and Apoptotic Cells

Immunohistochemical analyses were performed on cryostat-generated 20-μm sagittal brain sections mounted onto glass slides, as described by Daval and colleagues.[22] For Hcy immunostaining, tissue sections were incubated in 2 N HCl for 45 minutes at room temperature followed by a 10-minute wash in 0.1 mol/L sodium borate at pH 8.5. Slides were dipped in phosphate-buffered saline (PBS) for 10 minutes, then in PBS containing 10% bovine serum for 1 hour, and were incubated overnight at 4°C with a rabbit polyclonal antibody against Hcy diluted at 1/100 (Chemicon Int., Temecula, CA). Brain slices were then incubated for 1 hour at room temperature in the presence of a secondary antibody (anti-rabbit IgG conjugated to Alexa Fluor, 1/100; Molecular Probes, Cergy Pontoise, France).

To identify the phenotype of Hcy-positive cells, tissue sections were also processed for the immunological detection of NeuN (a neuronal marker) or glial fibrillary acid protein (GFAP, a marker for astrocytes). The experimental protocol was as described above by using a mouse monoclonal antibody against NeuN (1/100; Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal antibody against GFAP (1/200, Chemicon Int.) followed by the second-step antibody (anti-mouse IgG conjugated to Alexa Fluor, 1/1000; Molecular Probes).

For the specific detection of apoptosis, the method initially described by Frankfurt and Krishan[23] was used. Tissue sections previously fixed in 5% paraformaldehyde (4°C for 24 hours) were incubated in 50% formamide at 56°C for 30 minutes. After washing with phosphate buffer (PBS), tissue was blocked in PBS containing 10% bovine serum for 1 hour and then incubated overnight at 4°C with Apostain F7-26 monoclonal antibody (AbCys SA, Paris, France) specific to single-stranded DNA. Brain sections were then rinsed with PBS and incubated for 1 hour with Alexa Fluor-conjugated anti-mouse IgM (1/1000; Molecular Probes). According to the manufacturer, F7-26 monoclonal antibody can be used in combination with another antibody, and co-labeling experiments were conducted with Hcy antibody. A test with bovine serum albumin and Hcy antibody was previously done to ensure the specificity of Hcy antibody after tissue incubation in formamide at 56°C. In addition, expression of the apoptosis-related protein p53 was analyzed in brain sections by means of a rabbit monoclonal antibody against p53 diluted at 1/100 (Santa Cruz) and its corresponding secondary antibody (anti-rabbit IgG conjugated to Alexa Fluor; Molecular Probes). In all cases, control experiments were conducted by processing adjacent sections in the same way except that the primary antibody was omitted.

For quantification of specifically labeled cells, total cell density in definite brain areas was calculated after staining cell nuclei by the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml in PBS; Sigma), according to Wolvetang and colleagues.[24] The number of cell nuclei was scored at 365 nm under fluorescence microscopy (Axioscop; Zeiss, Strasbourg, France) at a $\times$40 magnification in at least six separate experiments by counting cells with their nuclei present in the focal plane in three distinct section areas delineated by an ocular grid of 1/400 mm^2, and the amounts of cells exhibiting any of the selective markers were calculated as a percentage of total cells. DAPI staining was also used at higher magnification for chromatin observation and detection of apoptosis hallmarks.

Assessment of Neurobehavioral Development

Righting Reflex

The static righting reflex was studied as described by Schroeder and colleagues.[25] The time needed by the pup to right itself in a supine position was recorded for 3 consecutive days (postnatal days 3 to 5).

Negative Geotaxis

This reflex was tested at postnatal days 8, 9, and 10. The rat was positioned with the head downward on an inclined plane with a 20% slope. The time needed for the pup to turn completely and reach a position with the head upward on the plane was measured. The duration of the test was limited to 120 seconds.[25]

Suspension Time

The test was performed at postnatal day 10. The rat pup was suspended by its front paws grasping a metal rod (1-mm diameter) that was stretched between the two poles of a frame at ~20 cm above the table. A Plexiglas sheet was placed in front of the rat to prevent it from turning around the rod. The time the animal remained on the bar was recorded. This test was performed before eyelid opening to control muscle strength by itself and eliminate the participation of emotivity.[25]

Locomotor Coordination

This test adapted by Schroeder and colleagues[25] was performed at postnatal days 19, 20, and 21. It is divided into three phases, and its total duration is limited to 300 seconds. During the first phase of the test, the rat is forced to swim in a round container (15-cm diameter and 23-cm height) half full of water. The animal is able to come out of the water by climbing along a metal rod (8-mm diameter) that is deep enough inside the water so

that the rat necessarily encounters it during the swimming phase. The second phase represents the climbing period along the rod (35 cm). During the third phase, the animal reaches a platform on which it can restore a normal quadruped position after the climbing task. The time necessary for the rat to succeed, ie, to reach the platform with the four paws after being put inside the water, was recorded. Respective times necessary to complete each of the three different phases of the test were also measured.

Elevated-Plus Maze

The elevated-plus maze is a validated test that evaluates anxiety in rodents.[26] The apparatus was made of transparent Plexiglas. It comprises two open arms (50 × 10 cm), two enclosed arms (10 × 40 × 50 cm), and a central platform (10 × 10 cm). The configuration has the shape of a plus sign, and the apparatus is elevated 80 cm above the floor level. Grip on the open arms is facilitated by inclusion of a small edge (0.5 cm high) around their perimeter. Rats were tested at 80 days. They were brought to the room 2 hours before the test and were tested individually. Before each trial, the maze was cleaned thoroughly with a 30% ethanol solution. At the beginning of the test, rats were placed on the central platform, always facing the same open arm. The test lasted 5 minutes in standard laboratory conditions under red light. The testing device was video-recorded, and the experimenter supervised the test in an adjacent room. Videotapes were scored by a naive trained observer by using a software developed in our laboratory. Behaviors were encoded afterward directly on a PC keyboard. Data were then transferred for statistical analysis.

Activity- and anxiety-related behaviors were assessed. Standard measures comprised the total number of arm entries (arm entry defined as all four paws entering an arm), the number of open- and closed-arm entries, and the time spent in different sections of the maze (open and closed arms and central platform). In addition to conventional measures, two specific behavioral measures were recorded: rearing frequency/duration and head dipping frequency (exploratory movement of head/shoulders over the sides of the maze).

Eight-Arm Maze

Rats were tested at 80 days of age for spatial orientation, learning and memory capacities in the eight-arm radial maze according to the procedure originally described by Olton and Samuelson.[27] Animals were food deprived to 85% of their body weight before testing and maintained at that weight during the 5 days of testing. The apparatus was a wooden gray, enclosed eight-arm radial maze with walls and entirely covered with a transparent top. Each of the arms (60 × 12 × 17 cm) projected from one side of an octagonal central platform measuring 50 cm in diameter. One food pellet (45 mg) was positioned at the far end of each arm. Before each trial, every arm of the maze was baited with a food pellet. Reinforcement was not replaced during the test. The whole apparatus was video-recorded from above under red light. At the beginning of the test, a white plastic cylinder (45 cm in diameter) was used to place the rats in the central platform. The cylinder was taken back, and animals were left in the maze until they had either entered all eight arms or until 15 minutes had elapsed, whichever occurred first. Placing all four paws inside an arm was recorded as an arm entrance. Times of arm entrances, the identity of each arm entered, and the serial order were recorded. All rats were tested for 5 consecutive days (one test per day).

Statistical Analyses

Data were prospectively collected and analyzed with Statview 5 software for Windows (SAS Institute, Berkley, CA). Reported as means ± SD, raw data were compared by using one-way analysis of variance with Fisher's test. Regarding behavioral studies, the distribution of the data deviated quite strongly from normality and variances were not equal. Therefore, nonparametric statistics were used,[28] and data were analyzed by χ^2 test and Mann-Whitney *U*-test. Univariate *z*-correlation analyses were used to evaluate the relationships between plasma concentrations of vitamin B12, folate, Hcy, and the time to goal in negative geotaxis (day 8) and locomotor coordination (day 21) tests as well as between body weight and scores achieved in behavioral tests. For all analyses, a P value <0.05 was considered to indicate statistical significance.

Results

Vitamin and Hcy Concentrations

In control rats, plasma concentrations of vitamin B12 and folate significantly decreased during development, whereas homocysteinemia progressively increased (Table 1). When pups were fed by dams receiving the deficient diet, plasma levels of vitamin B12 and folate were persistently reduced as compared with controls, and these observations were associated with increasing homocysteinemia. In contrast, the erythrocyte glutathione reductase activation coefficient value in rats subjected to the deficient diet (1.1 ± 0.4) was similar to that recorded in rats subjected to a normal diet (1.1 ± 0.3), suggesting the absence of vitamin B2 deprivation. In tissue homogenates issued from the whole brain, the deficient diet was associated with a significant reduction of folate concentration at 21 days of age, without concomitant alteration of vitamin B12 concentration. Correlatively, brain concentration of Hcy was significantly augmented in deficient rats. Although not shown in Table 1, regional investigations showed a substantial increase in the cerebellum ($P < 0.01$), whereas changes in the hippocampus remained not significant ($P = 0.069$). Finally, cerebral concentration of SAM was unaffected by the dietary conditions, whereas SAH concentration was significantly

Table 1. Effects of the Dietary Regimen on Concentrations of Homocysteine and Homocysteine Determinants in Developing Rats

	Normal diet			Deficient diet		
Age	Day 2	Day 5	Day 21	Day 2	Day 5	Day 21
Plasma Hcy (μmol/L)	2.4 ± 0.4	2.3 ± 0.3	6.8 ± 0.3†‡	5.1 ± 2.1	11.4 ± 0.7*†	13.3 ± 3.7*†‡
Plasma vitamin B12 (pmol/L)	5579.0 ± 1348.3	2344.5 ± 429.6†	1202.6 ± 161.5†‡	2276.0 ± 118.2*	1036.6 ± 525.5*†	333.6 ± 61.4*†‡
Plasma folate (nmol/L)	203.0 ± 44.6	145.3 ± 21.7†	86.9 ± 15.5†‡	180.2 ± 38.8	76.2 ± 21.8*†	33.1 ± 10.7*†‡
Brain Hcy (pmol/mg protein)	n.d.	n.d.	99.7 ± 9.8	n.d.	n.d.	133.8 ± 14.2*
Brain vitamin B12 (pmol/mg tissue)	n.d.	n.d	451.6 ± 169.8	n.d.	n.d.	416.6 ± 72.1
Brain folate (nmol/mg tissue)	n.d.	n.d.	70.4 ± 12.3	n.d.	n.d.	33.9 ± 8.4*
Brain SAM (nmol/g)	n.d.	n.d.	20.3 ± 5.5	n.d.	n.d.	22.0 ± 14.4
Brain SAH (nmol/g)	n.d.	n.d.	0.9 ± 0.4	n.d.	n.d.	2.0 ± 1.2*
SAM/SAH ratio	n.d.	n.d.	32.9 ± 6.9	n.d.	n.d.	11.8 ± 1.8*

Data are means ± SD and were obtained from six individuals at each developmental stage. Statistically significant differences ($P < 0.05$, analysis of variance): *between deficient diet and normal diet; †versus day 2; ‡versus day 5.

Hcy, homocysteine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; n.d., not determined.

enhanced by the early exposure to deficient diet. As a consequence, the brain SAM/SAH ratio was found to be markedly reduced in the deficient group (Table 1). In parallel, substantial growth retardation was recorded in rats early exposed to the deficient diet, as previously documented.[16] At postnatal day 21, body weight was reduced from 44.66 ± 3.13 g in controls to 17.72 ± 4.30 g in deficient animals ($n = 12$, $P < 0.0001$), whereas brain weight varied from 1.53 ± 0.08 g to 1.08 ± 0.03 g ($n = 12$, $P < 0.0001$).

Brain Accumulation of Homocysteine

As illustrated in Figure 1, Hcy immunoreactivity in deficient pups was dramatically increased in specific brain regions corresponding to the cerebellum, the hippocampus, the striatum (caudate nucleus and putamen), and the subventricular zone lining the lateral ventricle. In the cerebellum, strongest staining was detected in the granular cell layer. In the hippocampus, the CA1 pyramidal layer exhibited particularly high amounts of Hcy-reactive cells. In this structure, the percentage of total cells that were Hcy-positive was found to increase from 3.5 ± 1.5 in controls to 32.7 ± 9.1 ($P < 0.0001$) at 21 days of age. The density of Hcy-positive cells was lower in other hippocampal subregions, whereas the presence of Hcy was hardly detectable in the dentate gyrus and hilus. Such observations certainly account for the lack of significant changes in Hcy concentration in the total hippocampus. High magnification revealed that Hcy accumulated in the cytosol and was present in cell processes (Figure 2, A and B). Further immunohistochemical experiments performed with specific cell markers in combination with Hcy antibody revealed that Hcy was present in both neurons and astrocytes, as illustrated in Figure 2B showing the localization of Hcy in CA1 hippocampal cells expressing either the neuronal protein NeuN or the glial marker GFAP.

Induction of Apoptosis

At 21 days of age, immunological labeling of cells exhibiting single-stranded DNA (Apostain, a specific marker for apoptotic cells) showed a substantial increase (fivefold) of cells undergoing apoptosis in the CA1 hippocampal layer of rats exposed to the deficient diet as compared with controls (Figure 2C). In parallel, the number of cells expressing the well-known apoptosis-related protein p53 increased in the same proportion (Figure 2, D and E). Co-labeling studies allowed to show that a high number of cells containing Hcy were positive for the specific antibody against single-stranded DNA as well as for p53, suggesting the occurrence of Hcy-associated apoptotic cell death in the hippocampus of deficient rats.

The presence of apoptosis was further documented after DAPI staining by the identification of condensed chromatin and apoptotic bodies (Figure 2F). Coupled with immunolabeling of cell types, this procedure also showed that both neurons and astrocytes underwent apoptosis, but neurons appeared more prone to cell death (not shown). Finally, histological staining by thionine confirmed cell loss in rats subjected to the deficient regimen, as shown by an obvious reduction of the thickness of the CA1 pyramidal layer that reached 48.2% (Figure 2G). Immunostaining of GFAP or vimentin did not reveal a noticeable gliosis.

MS and CBS Analyses

Because cellular Hcy can be eliminated by either MS (remethylation pathway) or CBS (transsulfuration pathway), these two enzymes were analyzed at postnatal day 21 in brain regions where Hcy was shown to accumulate as a consequence of the deficient diet. Results are only illustrated by studies performed in the cerebellum (Figure 3),

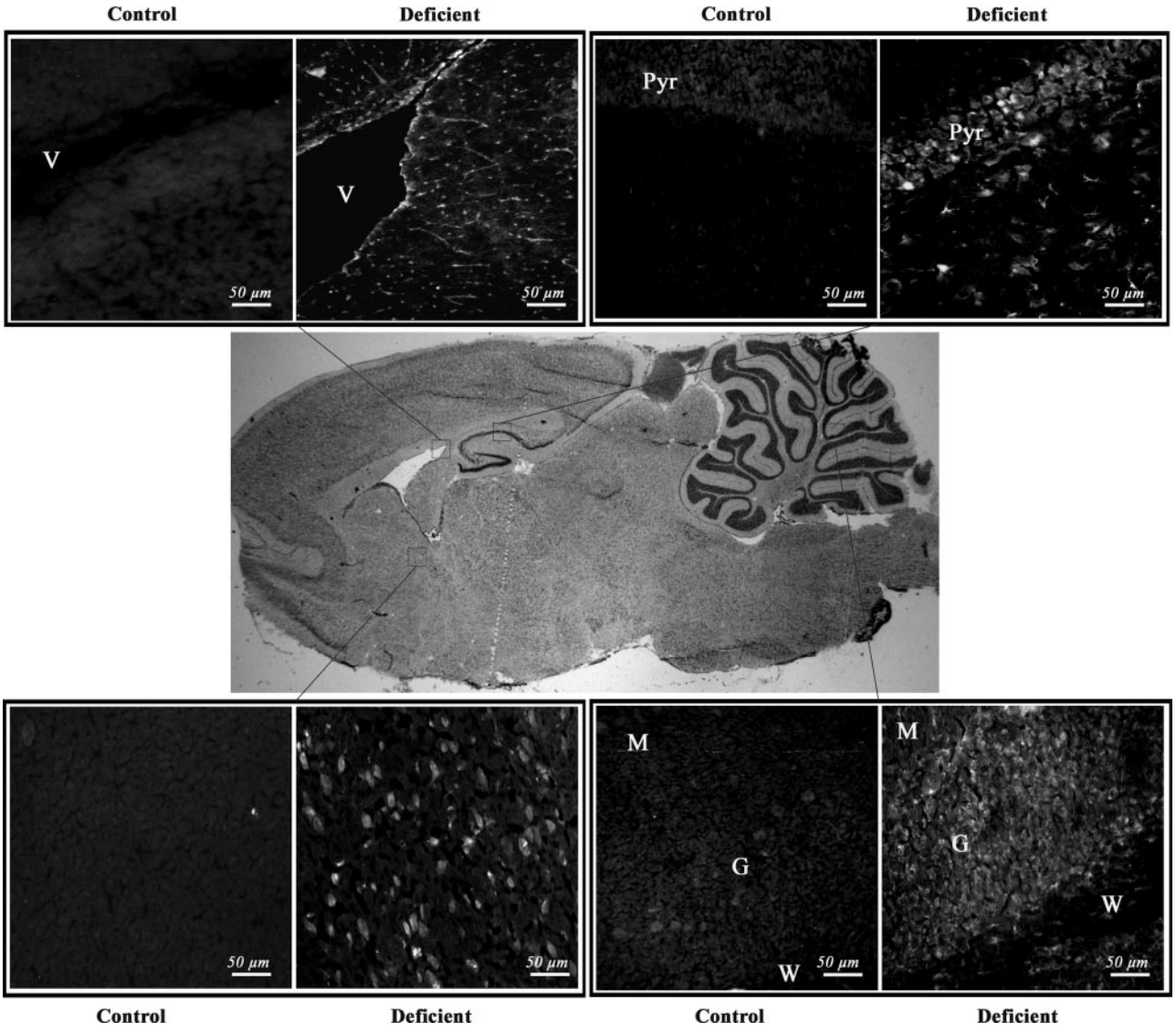

Figure 1. Brain distribution of Hcy at postnatal day 21 in control rats and those early exposed to the deficient diet. Immunostaining of homocysteine-positive cells was performed on sagittal brain sections by means of a specific antibody ($n = 6$ per group). In the deficient group, sustained accumulation of Hcy was observed in the brain structures shown, ie, the cerebellum (granular layer), the CA1 area of the hippocampus (pyramidal cell layer), the caudate nucleus, and the subventricular zone lining the lateral ventricle. V, ventricle; Pyr, pyramidal cell layer; M, molecular layer of the cerebellum; G, granular layer; W, white matter. Original magnifications, ×20.

and similar profiles were obtained in the other brain areas examined (not shown). Enzymatic activity of MS was dramatically altered by the nutritional conditions and varied in the cerebellar tissue from 18.9 ± 4.3 nmol/hour/mg in controls to 3.3 ± 0.4 nmol/hour/mg in deficient animals ($n = 8$, $P < 0.0001$). By contrast, CBS activity remained unaffected, corresponding to 10.9 ± 1.8 nmol/hour/mg in the deficient group versus 11.2 ± 1.9 nmol/hour/mg in the control group ($n = 8$, $P = 0.8376$) (Figure 3, left). In an attempt to test whether alterations of gene transcription might account for the above observations, mRNA levels were also quantified. As shown in Figure 3 (right), neither the amounts of MS mRNAs nor the amounts of CBS mRNAs in the rat cerebellum seemed to be modified by the early exposure to the deficient diet. Indeed, no significant differences could be found in *MS/L27* ratio values between the two experimental groups, and the same conclusion can be drawn about *CBS/L27* ratios.

Neurobehavioral Development

Righting Reflex, Negative Geotaxis, and Suspension Time

No statistically significant difference was found between the two experimental groups (ie, normal diet and deficient diet) in the percentage of animals that were able to achieve the tests evaluating righting reflex (postnatal days 3 to 5) and negative geotaxis (postnatal days 8 to 10) (Figure 4, A and B). However, it is noticeable that in the former test, the time necessary to

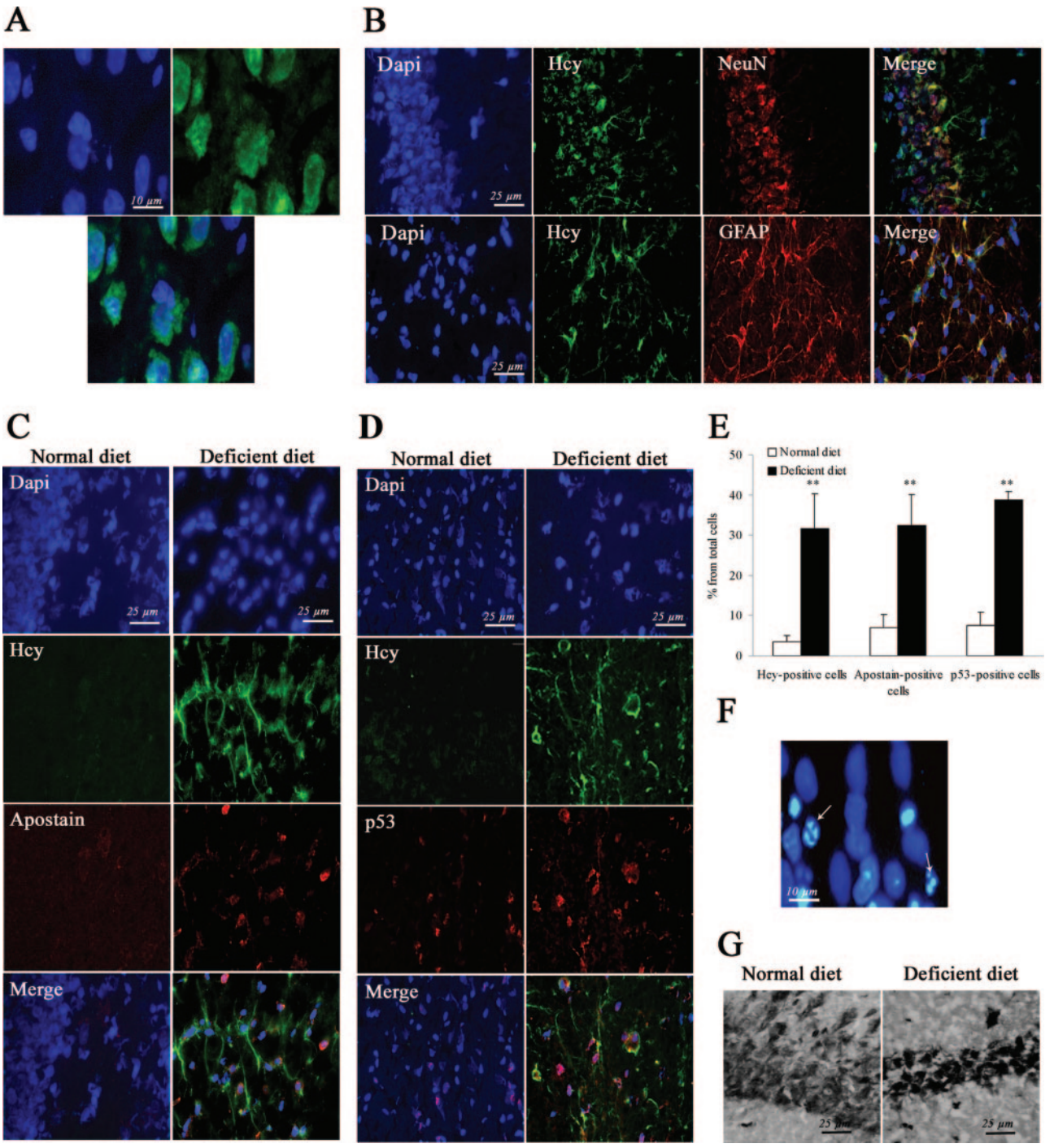

Figure 2. Immunohistochemical identification of homocysteine-containing cells and associated apoptosis in the CA1 hippocampus of 21-day-old rats exposed to the deficient regimen. **A:** Localization of Hcy in pyramidal cells counterstained with DAPI. **B:** Tissue sections were co-labeled with either a neuronal marker (NeuN) or a glial marker (GFAP). **C:** Apoptosis was analyzed by means of the Apostain F7-26-specific monoclonal antibody against single-stranded DNA. **D:** Immunohistochemical detection of Hcy and p53 in cells counterstained by DAPI. **E:** Amounts of cells positive to Hcy, apostain, and p53 in the CA1 pyramidal cell layer. Cell counts were performed in brain sections from seven different animals for both experimental groups. Data were calculated as a percentage from total cells (stained by DAPI) and reported as means ± SD. Statistically significant difference between the two experimental groups: $^{**}P < 0.0001$ (analysis of variance, Fishers test). **F: Arrows** designate apoptosis hallmarks as depicted by nuclear staining with DAPI. **G:** Thionin coloration of the CA1 hippocampus in control and deficient rats. Original magnifications: ×100 (**A**, **F**); ×40 (**B**, **D**, **G**).

come back to a quadruped position was transiently but significantly increased in the deficient group compared with the control group on the second day of testing (12.8 ± 2.7 versus 5.8 ± 0.9 seconds, $P < 0.05$). In the second test, the same observations were made for the time needed to turn up completely in the

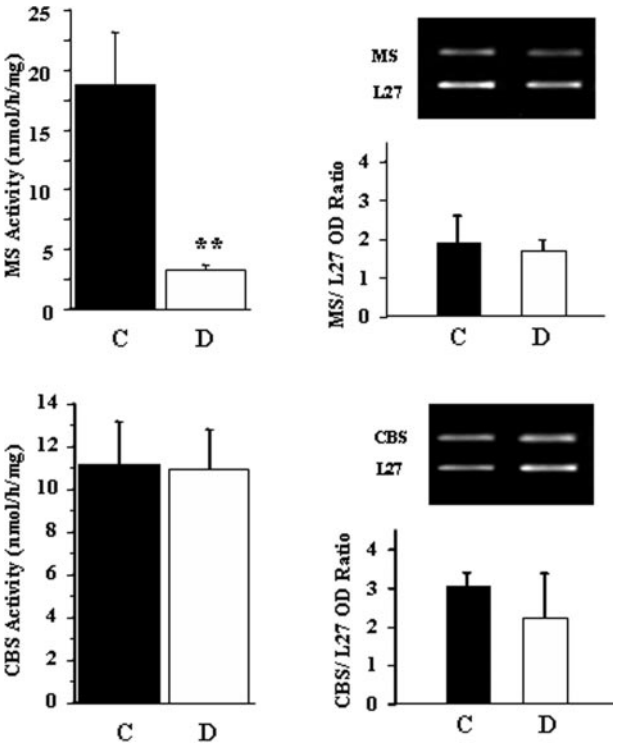

Figure 3. Effect of the deficient diet on MS and CBS activities ($n = 8$, left) and their mRNA levels ($n = 8$, right) in the cerebellum of 21-day-old rats. Values are means ± SD. C, control; D, deficient. Statistically significant difference between the two experimental groups: $^{**}P < 0.0001$ (analysis of variance).

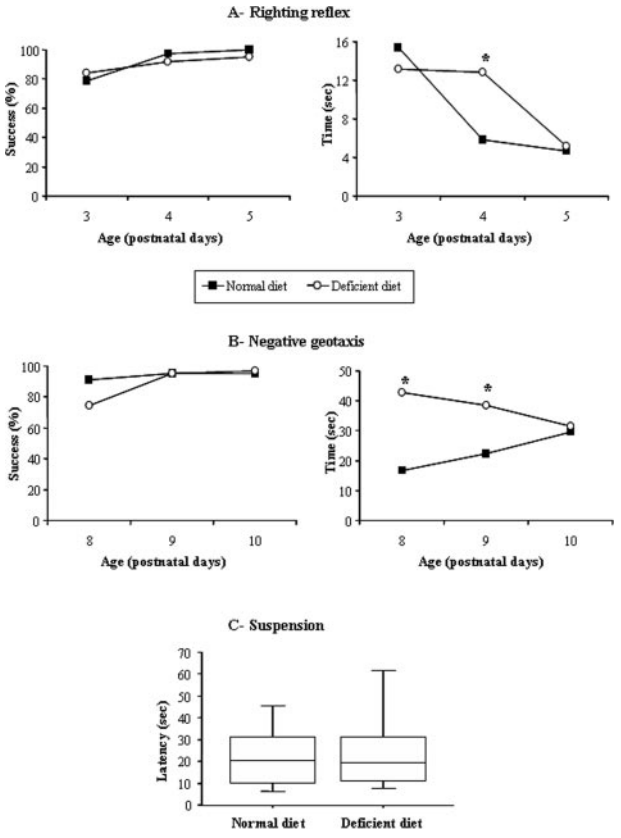

Figure 4. Effects of the dietary regimen on neurobehavioral development of rat pups ($n = 30$ per group). For righting reflex and negative geotaxis, score values represent percentages of successful animals (left) and median times (in seconds) for performing the test (right). Suspension times are given as medians and quartiles. Statistically significant differences between the two experimental groups: $^{*}P < 0.05$ (two-tailed Mann-Whitney *U*-test).

slope on both the 1st and 2nd days of testing ($P < 0.05$). Regarding suspension time at postnatal day 10, the median time during which rat pups were able to grasp the metal rod before falling was similar in the two experimental groups (Figure 4C).

Locomotor Coordination

As a whole, there was a statistically significant difference between the two animal groups in the global rates of success for the full test of locomotor coordination throughout the 3 days during which the experiment was repeated (Figure 5, top). On the 3rd day, all control animals were successful in achieving the task, whereas 37% of rats early exposed to the deficient diet were unable to perform the test. In addition, the time necessary to perform the whole part of the test was persistently longer in deficient rats. According to our data, the swimming phase mainly accounted for the globally poorer performances recorded in deficient pups (Figure 5). Indeed, the success score was substantially reduced in deficient rats, and the time spent to swim and to find the pole inside the water was systematically longer for deficient animals than for their control congeners. In all cases, differences between controls and deficient pups remained significant after correction of the data for body weight.

Correlation Studies

Plasma concentrations of Hcy were significantly correlated to concentrations of folate ($P < 0.0001$) and vitamin B12 ($P < 0.0001$) in 21-day-old rats. At this stage, brain weight was significantly correlated to plasma concentrations of folate ($P < 0.0001$), vitamin B12 ($P < 0.0001$), and Hcy ($P < 0.0001$). At the functional level, the time to goal in negative geotaxis at postnatal day 8 was significantly correlated to plasma concentrations of folate ($P = 0.0455$), vitamin B12 ($P = 0.0078$), and Hcy ($P = 0.0002$), whereas the time to achieve the full test of locomotor coordination at postnatal day 21 was correlated to plasma concentrations of vitamin B12 ($P = 0.0097$) and Hcy ($P < 0.0001$).

Consequences of Dietary Reversal at Weaning

After weaning, rat pups that received the deficient regimen *in utero* and then through their mothers' milk were then fed with standard food until postnatal day 80. To evaluate the long-term effects of the dietary reversal and to compare with control rats that received standard diet during the whole period, various parameters that are in line with Hcy metabolism were monitored again at 80 days of age. Table 2 shows that all factors studied

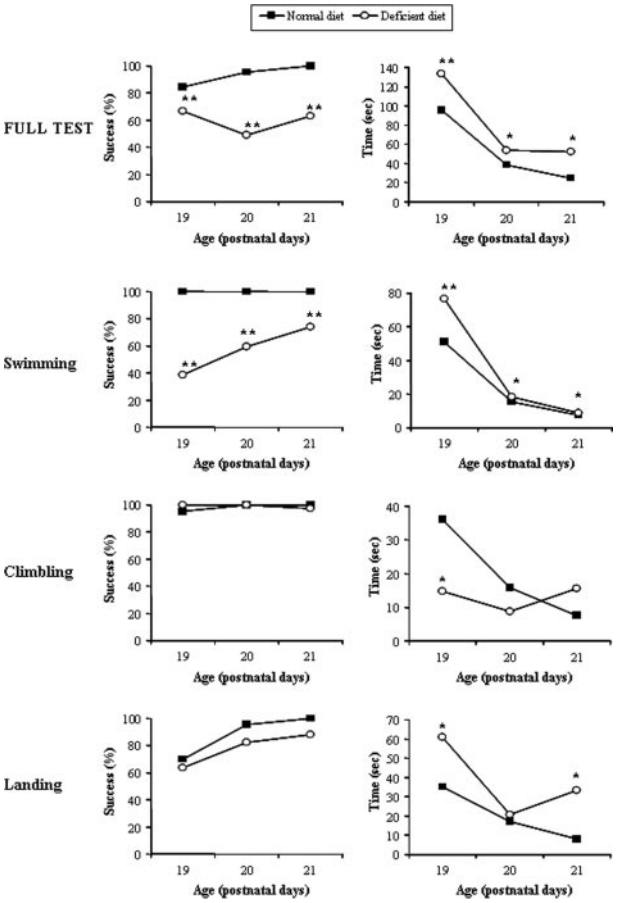

Figure 5. Effects of the dietary regimen on locomotor coordination evaluated at 19 to 21 days of age ($n = 35$ per group). Score values represent percentages of successful animals (left) and median times (in seconds) for performing the whole test and to achieve the various phases of the test (right). Statistically significant difference between the two experimental groups: $^{*}P < 0.05$ and $^{**}P < 0.0001$ (two-tailed Mann-Whitney *U*-test).

seemed to return to basal values within 2 months after the switch of dietary regimen to normal food. In particular, plasma and brain concentrations of Hcy were similar in the two experimental groups. Only plasma levels of folate were slightly more elevated in the previously deficient group compared with controls ($P = 0.0413$). It is noteworthy, however, that body weight and brain weight remained significantly altered at postnatal day 80. Indeed, the recorded mean body weight was 306.60 ± 13.52 g versus 373.80 ± 22.08 g in controls ($n = 12$, $P = 0.0004$), and mean brain weight was found to be 1.81 ± 0.93 g versus 2.07 ± 0.13 g ($n = 12$, $P = 0.0076$). Moreover, thionine staining depicted long-lasting histological deficits in the CA1 hippocampus (Figure 6), whereas no patent morphological alterations could be seen in other brain regions, at least with the methods used.

Table 2. Effect of Dietary Reversal to Normal Food at Weaning on Concentrations of Homocysteine and Its Determinants in 80-Day-Old Rats

	Control group ($n = 5$)	Deficient group ($n = 5$)
Plasma Hcy (μmol/L)	10.0 ± 0.6	8.4 ± 1.7
Plasma vitamin B12 (pmol/L)	727.4 ± 95.5	837.2 ± 55.6
Plasma folate (nmol/L)	144.2 ± 59.8	232.2 ± 54.7*
Brain Hcy (nmol/mg protein)	431.3 ± 72.6	357.7 ± 97.3
Brain vitamin B12 (pmol/mg)	363.4 ± 51.9	341.6 ± 27.5
Brain folate (nmol/mg)	73.2 ± 22.6	80.8 ± 9.3
Brain MS activity (nmol/hour/mg)	6.0 ± 2.6	5.0 ± 2.6
Brain CBS activity (nmol/hour/mg)	3.8 ± 0.4	3.6 ± 0.7

Data are means ± SD. Statistically significant difference between the two experimental groups: $^{*}P < 0.05$ (analysis of variance).

Hcy, homocysteine; MS, methionine synthase; CBS, cystathionine β-synthase.

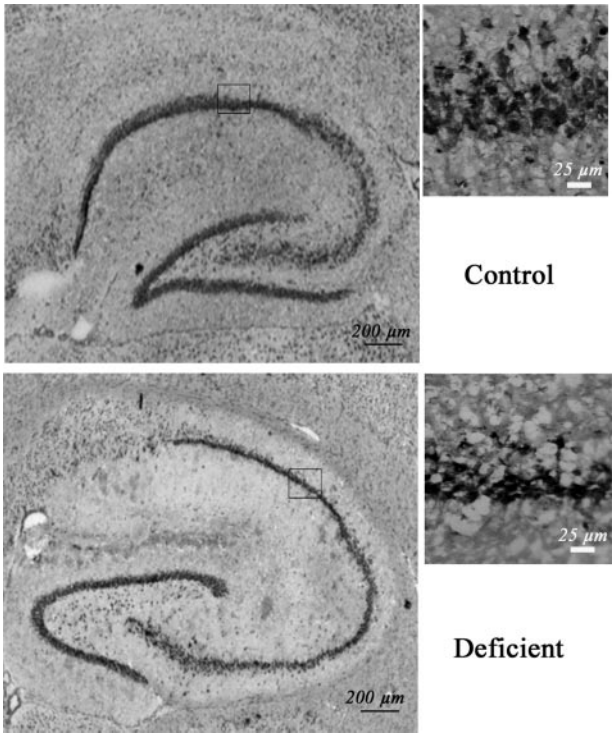

Figure 6. Thionin coloration of the CA1 hippocampus in control and previously deficient rats at 80 days of age. Original magnifications, ×40.

Exploratory Behavior in the Elevated-Plus Maze

The number of total arm entries as well as the percentage of entries in open arms was similar in the two experimental groups (Table 3). In addition, the time spent in open arms and in protected areas (which include closed arms and the central platform) did not differ between previously deficient rats and controls. The number of rearings and rearing duration were significantly lower in the deficient group ($P = 0.043$ and $P = 0.012$, respectively). More specifically, rearing duration was reduced both on open arms ($P = 0.039$) and in protected areas ($P = 0.022$), and it is noteworthy that rearings occurred more frequently in protected areas ($P = 0.009$) and less frequently in open arms ($P = 0.009$). Finally, head dipping over the sides of the maze, another aspect of vertical exploratory behavior, was also altered in the deficient group, its frequency being significantly reduced in open

Table 3. Effect of Early Exposure to the Deficient Diet Followed by Dietary Reversal to Normal Food at Weaning on Specific Behaviors in the Elevated-Plus Maze in 80-Day-Old Rats

Behavioral items	Control group (n = 14)	Deficient group (n = 22)
Total number of arm entries	27.5 (8)	26 (8)
Percent entries on open arms	20 (5.2)	22 (9.1)
Percent entries in protected areas	80 (4.0)	79.6 (8.7)
Time spent on open arms (seconds)	159.1 (54.6)	135.7 (85.8)
Time spent in protected areas (seconds)	140.9 (54.6)	164.3 (87.7)
Total number of rearings	16.5 (10)	12 (10)*
Percent rearings on open arms	17.2 (20.7)	5.3 (13.5)†
Percent rearings in protected areas	82.8 (20.7)	94.7 (13.5)†
Total rearing duration	26.4 (15.5)	18.3 (11.2)*
Rearing duration on open arms (seconds)	3.1 (2.7)	0.7 (4)*
Rearing duration in protected areas (seconds)	23.2 (20)	16.0 (11.5)*
Total number of head dips	17.0 (4.5)	16.0 (8.0)
Percent head dips on open arms	79.9 (20.8)	71.3 (20.1)*
Percent head dips in protected areas	20.1 (15.5)	28.7 (20.1)*

Values correspond to medians with interquartiles in parentheses. Statistically significant difference between the two experimental groups: *$P < 0.05$ and †$P < 0.01$ (two-tailed Mann-Whitney U-test). Protected areas include closed arms and the central platform.

arms (P = 0.018) and augmented in closed arms (P = 0.018) (Table 3).

Learning and Memory Behavior (the Eight-Arm Maze)

In the control group, the total time necessary to enter all eight arms of the maze progressively decreased throughout the 5 days of testing (P = 0.001 between day 1 and day 5, Wilcoxon's test) (Figure 7A). In parallel, the total number of arms visited per session and the total number of errors per session fell starting from the 4th day of testing (Figure 7, B and C). Because the deficient group needed approximately the same time to complete the task during the five consecutive sessions, the total number of arms visited per session and the corresponding number of errors markedly decreased between the 1st and the 2nd days of testing to finally remain stable (Figure 7, B and C). For the latter two parameters, the score was significantly different between the two experimental groups during sessions two and three, inasmuch as the total number of arms visited and the number of errors during these two sessions were inferior in deficient animals. Finally, the total number of arms entered per minute was lower in the deficient group than in the control group during sessions two, four, and five, suggesting a reduced locomotor activity (Figure 7D).

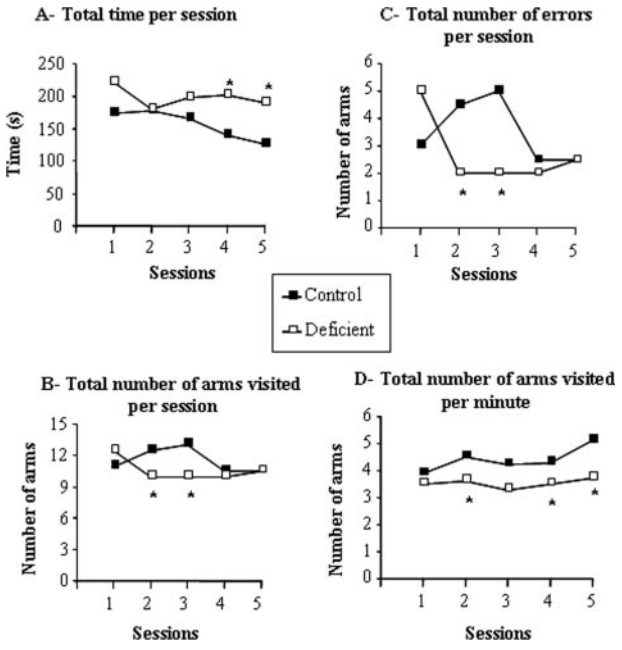

Figure 7. Effect of early exposure to the deficient diet followed by dietary reversal to normal food at weaning on the rat behavior in the eight-arm maze at postnatal day 80. Data represent median values from 14 control rats and 22 rats previously exposed to deficient diet. Statistically significant difference between the two experimental groups: *$P < 0.05$ (Mann-Whitney U-test).

Discussion

In line with elevated homocysteinemia, reduced intake of vitamins B, mainly folate and vitamin B12, has been implicated in several neurological and psychological disorders. Hcy has been associated with cognitive dysfunctions in Down syndrome and in the elderly, and it has been identified as a predictor of Alzheimer's disease.[3,5,29–32] Our study showed that gestational vitamin B deficiency leads to accumulation of Hcy with concomitant apoptosis in selective brain areas and persistently alters neurobehavioral capacities in developing rats.

Physiologically, a progressive increase in Hcy plasma concentration was documented between birth and weaning in control animals. As previously reported, this observation can be attributed to developmental changes in the activities of hepatic enzymes involved in Hcy metabolism.[33] At each developmental stage studied, the deficient diet resulted in a significant reduction of plasma concentrations in vitamin B12 and folate, with no biological evidence of vitamin B2 depletion, considering the erythrocyte glutathione reductase activation coefficient values. As a consequence, homocysteinemia was markedly augmented to reach values corresponding to clinically recognized moderate hyperhomocysteinemia in humans.[16]

In the brain tissue, early exposure to the deprived regimen was associated at 21 days of age with a lower

folate concentration without concomitant alteration of vitamin B12 concentration, probably reflecting a sparing process. As expected, deficient rats displayed a noticeable brain accumulation of Hcy. In parallel, brain concentration of SAH was substantially elevated, whereas SAM concentration remained unaffected. This may be attributable to the reversed activity of the bi-directional enzyme SAH hydrolase in response to increased Hcy levels. Because SAH is itself a potent competitive inhibitor of SAM-mediated methylation reactions, the resulting fall of the SAM/SAH ratio can therefore affect DNA methylation and thus alter gene expression, as previously shown in rat hepatocytes.[34] Immunohistochemical analyses revealed that Hcy was not distributed uniformly in the brain but accumulated in specific regions, including the cerebellum (predominantly in the granular cell layer), the hippocampus (mostly in the CA1 pyramidal layer), the striatum (in the caudate-putamen), and the subventricular zone lining the lateral ventricle. These observations are in good accordance with a pioneering study by Chung and colleagues[35] using transgenic mice expressing a human Cu/Zn SOD mutation that reported high amounts of Hcy in the hippocampal CA1 layer and in cerebellar nuclei. Likewise, Hcy was found to accumulate in the same brain areas in $CBS^{-/-}$ mice, another model of hyperhomocysteinemia, in which the authors also reported the presence of Hcy in Purkinje cells and basket cells in cerebellum.[36] In addition, we showed the presence of Hcy in the subventricular zone, which is known as a neurogenic site in the adult mammalian brain.[37] This observation is of interest regarding the brain capacity to generate new cells inasmuch as it was recently reported that folate deficiency can inhibit proliferation of progenitors in the adult mouse brain.[38] Regional examination of two key enzymes involved in Hcy metabolism, ie, MS and CBS, revealed that the transsulfuration pathway, reflected by CBS activity, was not affected in the brain of deficient rats, by contrast to the severe alteration previously observed in the liver.[16] Conversely, MS activity was dramatically reduced in deficient pups as compared with controls (eg, it was approximately sixfold less active in the cerebellar tissue of deficient rats than in controls), suggesting that the remethylation pathway may play a critical role in brain accumulation of Hcy in our model. According to our data, alteration of MS activities observed in the deficient group cannot be explained by changes in gene transcription. Reduced availability of folate, a co-substrate for MS, and possibly of circulating vitamin B12 probably account for the decrease in enzyme activity. Furthermore, it has been reported that MS activity is mainly regulated at the post-transcriptional level.[39,40]

Co-labeling studies showed that Hcy was present in both neuronal cells and astrocytes of deficient rats. Indeed, Hcy-positive cells expressed either NeuN or GFAP that are specific markers for neurons and astrocytes, respectively. Both types of neural cells have been reported to potentially accumulate Hcy.[15,41,42] Until very recently, it was considered that only neurons, but not astrocytes, possessed sufficient amounts of the enzyme CBS to catabolize Hcy,[43] and it was suggested that astrocytes need to export Hcy to neurons to keep low levels of the amino acid, while they take up cysteine.[44] According to Benz and colleagues,[45] such a process could influence the functional crosstalk between neurons and astrocytes. It must be noticed, however, that it has been newly demonstrated that CBS is significantly expressed in astrocytes throughout the mouse brain.[46] In the hippocampus of deficient rats, apoptosis was found in cells containing Hcy, in line with an increased expression of p53, one major step through which Hcy has been shown to promote apoptotic cell death.[3] Hcy-induced neurotoxicity is known to also include DNA damage and altered DNA repair attributable to disturbance in the DNA methylation cycle, glutamate excitotoxicity via stimulation of *N*-methyl-D-aspartate (NMDA) receptors, endoplasmic reticulum stress, calcium overload, and oxidative stress.[3,47]

Clinically, it is known that elevated levels of Hcy are linked to neurological impairments and cognitive decline.[3,6,48] Accordingly, the present study reports that neurobehavioral development was altered in hyperhomocysteinemic rats. Data recorded in early tests (ie, righting reflex and negative geotaxis) certainly reflect delayed brain maturation,[49] whereas the lack of difference in the suspension time between the two experimental groups is in favor of the absence of muscular weakness in deficient rats, in agreement with their harmonious growth retardation, as previously documented.[16] At 19 to 21 days of age, global scores for achieving the test of locomotor coordination were significantly reduced after early exposure to the deficient regimen. Both the success scores and the time necessary to perform the tasks were affected in deficient rats, indicating poorer locomotor capacities. There were no correlations between body weight and behavioral scores. It can be hypothesized that the accumulation of Hcy recorded in motor areas such as the cerebellum and the striatum may account, at least partly, for these observations. It has been recently shown that experimentally induced increase of Hcy amounts in striatal regions decreased dopamine concentrations and reduced locomotor activity in mice and rats.[50]

To our knowledge, no study has investigated the potential reversibility of hyperhomocysteinemia effects in a nutritional animal model. When deficient pups were fed with standard food after weaning, homocysteinemia returned to normal values. Brain levels of folate, Hcy, as well as MS enzymatic activity were no longer different from controls. In the elevated plus-maze, global exploration of open and closed arms was similar in the two experimental groups. Nevertheless, detailed analysis revealed that the previously deficient group exhibited a reduced vertical exploratory behavior as compared with controls. This was reflected by significant decreases in the total number and duration of rearings. In addition, both rearing and head-dipping frequencies were significantly reduced in open arms and augmented in secure areas. According to several authors,[51–53] such postural elements in the elevated plus-maze would be indicative of risk assessment, and our observations may reflect lower propensity to take risks rather than anxiety itself. This is in good agreement with behavioral scores re-

corded in the eight-arm maze where previously deficient rats, by contrast to controls, needed a rather constant time throughout the five sessions to visit all arms, suggesting impaired spatial memory. Nonetheless, such slowness was associated with a lower number of errors compared with controls as soon as the second day of testing. It can therefore be speculated that rats of the deficient group are more circumspect before entering an arm, need more time for risk assessment and decision making, to finally commit fewer errors than controls. The hippocampus has been reported to be prominently involved in spatial working memory, and the integrity of CA1 and CA3 pyramidal neurons, including their interconnections, is known to be of critical importance in learning and retrieval of spatial memory,[54] suggesting that the observed Hcy-associated cell apoptosis may have directly, and durably, affected the capacities of deficient rats. In addition, Hcy is known to act on glutamatergic NMDA and metabotropic receptors that are crucial for spatial learning and neuronal plasticity.[55]

In conclusion, the present study provides new insights to the brain response to early deprivation in methyl donors and thus to the resulting shortage of Hcy metabolism. The data suggest the occurrence of long-term functional disabilities, even in case of subsequent dietary normalization. Although a direct effect of folate and vitamin B12 deficiency cannot be excluded, for example via SAM depletion, which can result in decreased DNA methylation and enhanced DNA damage, or via increased synthesis of tumor necrosis factor-α related to vitamin B12 deprivation,[3,56,57] our observations may reflect that Hcy-mediated apoptotic cell death in sensitive brain areas involved in motor functions (striatum, cerebellum) or in learning and memory (hippocampus), whereas the generation of new neural cells might additionally be compromised by the dietary conditions. Therefore, authorities should be aware of the potential risk incurred by infants from women exposed during their pregnancies to a dietary regimen deprived in methyl donors, especially folate and vitamin B12.

Acknowledgment

We thank Sandra Audonnet-Blaise for assistance.

References

1. Finkelstein JD: The metabolism of homocysteine: pathways and regulation. Eur J Pediatr 1998, 157(Suppl 2):S40–S44
2. Refsum H, Ueland PM, Nygard O, Vollset SE: Homocysteine and cardiovascular disease. Annu Rev Med 1998, 49:31–62
3. Mattson MP, Shea TB: Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. Trends Neurosci 2003, 26:137–146
4. Bosco P, Guéant-Rodriguez RM, Anello G, Romano A, Namour B, Spada RS, Caraci F, Tringali G, Ferri R, Guéant JL: Association of IL-1 RN*2 allele and methionine synthase 2756 AA genotype with dementia severity of sporadic Alzheimer's disease. J Neurol Neurosurg Psychiatry 2004, 75:1036–1038
5. Guéant JL, Anello G, Bosco P, Guéant-Rodriguez RM, Romano A, Barone C, Gérard P, Romano C: Homocysteine and related genetic polymorphisms in Down's syndrome IQ. J Neurol Neurosurg Psychiatry 2005, 76:706–709
6. Herrmann W: The importance of hyperhomocysteinemia as a risk factor for diseases: an overview. Clin Chem Lab Med 2001, 39:666–674
7. Hague WM: Homocysteine and pregnancy. Best Pract Res Clin Obstet Gynaecol 2003, 17:459–469
8. Guéant JL, Guéant-Rodriguez RM, Anello G, Bosco P, Brunaud L, Romano C, Ferri R, Romano A, Candito M, Namour B: Genetic determinants of folate and vitamin B12 metabolism: a common pathway in neural tube defect and Down syndrome? Clin Chem Lab Med 2003, 41:1473–1477
9. Botto LD, Lisi A, Robert-Gnansia E, Erickson JD, Vollset SE, Mastroiacovo P, Botting B, Cochi G, de Vigan C, de Walle H, Feijoo M, Irgens LM, McDonnell B, Merlob P, Ritvanen A, Scarano G, Siffel C, Metneki J, Stoll C, Smithells R, Goujard J: International retrospective cohort study of neural tube defects in relation to folic acid recommendations: are the recommendations working? Obstet Gynecol Surv 2005, 60:563–565
10. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE: Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci 2001, 22:195–201
11. Wilcken B, Bamforth F, Li Z, Zhu H, Ritvanen A, Renlund M, Stoll C, Alembik Y, Dott B, Czeizel AE, Gelman-Kohan Z, Scarano G, Bianca S, Ettore G, Tenconi R, Bellato S, Scala I, Mutchinick OM, Lopez MA, de Walle H, Hofstra R, Joutchenko L, Kavteladze L, Bermejo E, Martinez-Frias ML, Gallagher M, Erickson JD, Vollset SE, Mastroiacovo P, Andria G, Botto LD: Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. J Med Genet 2003, 40:619–625
12. Amouzou EK, Chabi NW, Adjalla CE, Rodriguez-Guéant RM, Feillet F, Villaume C, Sanni A, Guéant JL: High prevalence of hyperhomocysteinemia related to folate deficiency and the 677 C → T mutation of the gene encoding methylenetetrahydrofolate reductase in coastal West Africa. Am J Clin Nutr 2004, 79:619–624
13. Chéry C, Barbe F, Lequere C, Abdelmouttaleb I, Gérard P, Barbarino P, Boutroy JL, Guéant JL: Hyperhomocysteinemia is related to a decreased blood level of vitamin B12 in the second and third trimester of normal pregnancy. Clin Chem Lab Med 2002, 40:1105–1108
14. Kruman II, Culmsee C, Chan SL, Fruman Y, Guo Z, Penix L, Mattson MP: Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J Neurosci 2000, 20:6920–6926
15. Maler JM, Seifert W, Huther G, Wiltfang J, Ruther E, Kornhuber J, Bleich S: Homocysteine induces cell death of rat astrocytes in vitro. Neurosci Lett 2003, 347:85–88
16. Blaise S, Alberto JM, Nédélec E, Ayav A, Pourié G, Bronowicki JP, Guéant JL, Daval JL: Mild neonatal hypoxia exacerbates the effects of vitamin-deficient diet on homocysteine metabolism in rats. Pediatr Res 2005, 57:777–782
17. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72:248–254
18. Ayav A, Alberto JM, Barbe F, Brunaud L, Gérard P, Merten M, Guéant JL: Defective remethylation of homocysteine is related to decreased synthesis of coenzymes B2 in thyroidectomized rats. Amino Acids 2005, 28:37–43
19. Taoka S, Ohja S, Shan X, Kruger WD, Banerjee R: Evidence for heme-mediated redox regulation of human cystathionine beta-synthase activity. J Biol Chem 1998, 273:25179–25184
20. Chen Z, Chakraborty S, Banerjee R: Demonstration that mammalian methionine synthases are predominantly cobalamin-loaded. J Biol Chem 1995, 270:19246–19249
21. Delabar U, Kloor D, Luippold G, Muhlbauer B: Simultaneous determination of adenosine, S-adenosylhomocysteine and S-adenosylmethionine in biological samples using solid-phase extraction and high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 1999, 724:231–238
22. Daval JL, Pourié G, Grojean S, Lièvre V, Strazielle C, Blaise S, Vert P: Neonatal hypoxia triggers transient apoptosis followed by neurogenesis in the rat CA1 hippocampus. Pediatr Res 2004, 55:561–567
23. Frankfurt OS, Krishan A: Identification of apoptotic cells by form-

amide-induced DNA denaturation in condensed chromatin. J Histochem Cytochem 2001, 49:369–378
24. Wolvetang EJ, Johnson KL, Krauer K, Ralph SJ, Linnane AW: Mitochondrial respiratory chain inhibitors induce apoptosis. FEBS Lett 1994, 339:40–44
25. Schroeder H, Humbert AC, Koziel V, Desor D, Nehlig A: Behavioural and metabolic consequences of neonatal exposure to diazepam in developing rats. Exp Neurol 1995, 131:53–63
26. Pellow S, Chopin P, File SE, Briley M: Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 1985, 14:149–67
27. Olton DS, Samuelson RJ: Remembrance of places passed: spatial memory in rats. J Exp Psychol Anim Behav Proc 1976, 2:97–116
28. Winer RI: Statistical Principles in Experimental Design. New York, McGraw-Hill, 1971
29. Riggs KM, Spiro III A, Tucker K, Rush D: Relations of vitamin B-12, vitamin B-6, folate, and homocysteine to cognitive performance in the Normative Aging Study. Am J Clin Nutr 1996, 63:306–314
30. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PW, Wolf PA: Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002, 346:476–483
31. Miller JW: Homocysteine, Alzheimer's disease, and cognitive function. Nutrition 2000, 16:675–677
32. McCaddon A, Regland B: Homocysteine and cognition—no longer a hypothesis? Med Hypotheses 2006, 66:682–683
33. Okada G, Watanabe Y, Tsukada K: Changes in patterns of S-adenosylmethionine synthetases in fetal and postnatal rat liver. Cancer Res 1980, 40:2895–2897
34. García-Trevijano ER, Martinez-Chantar ML, Latasa MU, Mato JM, Avila MA: NO sensitizes rat hepatocytes to proliferation by modifying S-adenosylmethionine levels. Gastroenterology 2002, 122:1355–1363
35. Chung YH, Hong JJ, Shin CM, Joo KM, Kim MJ, Cha CI: Immunohistochemical study on the distribution of homocysteine in the central nervous system of transgenic mice expressing a human Cu/Zn SOD mutation. Brain Res 2003, 967:226–234
36. Robert K, Santiard-Baron D, Chasse JF, Paly E, Aupetit J, Kamoun P, London J, Janel N: The neuronal SAPK/JNK pathway is altered in a murine model of hyperhomocysteinemia. J Neurochem 2004, 89:33–43
37. Kokaia Z, Lindvall O: Neurogenesis after ischaemic brain insults. Curr Opin Neurobiol 2003, 13:127–132
38. Kruman II, Mouton PR, Emokpae Jr R, Cutler RG, Mattson MP: Folate deficiency inhibits proliferation of adult hippocampal progenitors. Neuropharmacol Neurotoxicol 2005, 16:1055–1059
39. Yamada K, Kawata T, Wada M, Isshiki T, Onoda J, Kawanishi T, Kunou A, Tadokoro T, Tobimatsu T, Maekawa A, Toraya T: Extremely low activity of methionine synthase in vitamin B-12-deficient rats may be related to effects on coenzyme stabilization rather than to changes in coenzyme induction. J Nutr 2000, 130:1894–1900
40. Oltean S, Banerjee R: Nutritional modulation of gene expression and homocysteine utilization by vitamin B12. J Biol Chem 2003, 278:20778–20784
41. Duan W, Ladenheim B, Cutler RG, Kruman II, Cadet JL, Mattson MP: Dietary folate deficiency and elevated homocysteine levels endanger dopaminergic neurons in models of Parkinson's disease. J Neurochem 2002, 80:101–110
42. Ho PI, Ashline D, Dhitavat S, Ortiz D, Collins SC, Shea TB, Rogers E: Folate deprivation induces neurodegeneration: roles of oxidative stress and increased homocysteine. Neurobiol Dis 2003, 14:32–42
43. Robert K, Vialard F, Thiery E, Toyama K, Sinet PM, Janel N, London J: Expression of the cystathionine beta synthase (CBS) gene during mouse development and immunolocalization in adult brain. J Histochem Cytochem 2003, 51:363–371
44. Huang G, Dragan M, Freeman D, Wilson JX: Activation of catechol-O-methyltransferase in astrocytes stimulates homocysteine synthesis and export to neurons. Glia 2005, 51:47–55
45. Benz B, Grima G, Do KQ: Glutamate-induced homocysteic acid release from astrocytes: possible implication in glia-neuron signaling. Neuroscience 2004, 124:377–386
46. Enokido Y, Suzuki E, Iwasawa K, Namekata K, Okazawa H, Kimura H: Cystathionine β-synthase, a key enzyme for homocysteine metabolism, is preferentially expressed in the radial glia/astrocyte lineage of developing mouse CNS. FASEB J 2005, 19:1854–1856
47. Ho PI, Ortiz D, Rogers E, Shea TB: Multiple aspects of homocysteine neurotoxicity: glutamate excitotoxicity, kinase hyperactivation and DNA damage. J Neurosci Res 2002, 70:694–702
48. Bottiglieri T, Hyland K: S-Adenosylmethionine levels in psychiatric and neurological disorders: a review. Acta Neurol Scand 1994, 154:S19–S26
49. Grojean S, Schroeder H, Pourié G, Charriaut-Marlangue C, Koziel V, Desor D, Vert P, Daval JL: Histopathological alterations and functional brain deficits after transient hypoxia in the newborn rat pup: a long term follow-up. Neurobiol Dis 2003, 14:265–278
50. Lee ES, Chen H, Soliman KF, Charlton CG: Effects of homocysteine on the dopaminergic system and behaviour in rodents. Neurotoxicology 2005, 26:361–371
51. Cruz APM, Frei F, Graeff FG: Ethopharmacological analysis of rat behaviour on the elevated plus-maze. Pharmacol Biochem Behav 1994, 49:171–176
52. Rodgers RJ, Johnson NJT: Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav 1995, 52:297–303
53. Weiss SM Wadsworth G, Fletcher A, Dourish CT: Utility of ethological analysis to overcome locomotor confounds in elevated maze models of anxiety. Neurosci Biobehav Rev 1998, 23:265–271
54. Stubley-Weatherly L, Harding JW, Wright JW: Effects of discrete kainic acid-induced hippocampal lesions on spatial and contextual learning and memory in rats. Brain Res 1996, 716:29–38
55. Riedel G, Platt B, Micheau J: Glutamate receptor function in learning and memory. Behav Brain Res 2003, 140:1–47
56. Wainfan E, Poirier LA: Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 1992, 52:S2071–S2077
57. Scalabrino G, Peracchi M: New insights into the pathophysiology of cobalamin deficiency. Trends Mol Med 2006, 12:247–254